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Supplemental Data

The TRIM-NHL Protein TRIM32 Activates MicroRNAs and Prevents Self-Renewal in Mouse Neural Progenitors

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Supplemental Experimental Procedures

Materials and Plasmids

The following plasmids were used: pEGFP-N1 (Clontech), pGL3-Firefly (Promega), EGFP-TRIM32 (Reymond et al., 2001), c-myc (Gregory et al., 2005), HA-Ubiquitin (Treier et al., 1994), pll3.7 with or without coding sequence for Let-7a (Judy Lieberman, Boston), Luciferase vectors pRL-let-7a (Schmitter et al., 2006), pGL3-miR138. The TRIM32 ubiquitin-ligase mutation (TRIM32 C24A) was introduced with the quick-change site directed mutagenesis kit (Stratagen). For expression of shRNAs directed against TRIM32 the pSHAG-Magic2c vector (Open Biosystems) was used (functional sequence #4: GATCTTCAGGCAAGGTATA; functional sequence #12: CCCTGACGGTGCTGAAGATCAT; non-functional sequence #2: CTGACGGTGCTGAAGATCA). To inhibit the micro RNAs-138 and Let-7a specific locked nucleotide acids (LNAs, Exiqon) were used.

Proteasomal activity was inhibited with clasto-Lactacystin-β-lactone (Calbiochem).

The following primary antibodies were used: anti- Phospho-Histone H3 (P-H3) (Cell Signaling Technology), anti-Ki67 (Novo Castra), anti-Fibrillarin (Abcam), anti-Cleaved-Caspase 3 (Cell Signaling Technology), anti-TuJ1 (Covance), anti-Nestin (BD Biosciences), anti-c-Myc (Santa Cruz), anti-MAP2 (Chemicon), anti-HA (Roche), anti-Alpha-Tubulin (Sigma), anti-Hsp70 (Abcam), anti-Gamma-Tubulin (Sigma), anti-TRIM32 (Abnova), anti-Ago1 (Gunter Meister, Munich). As secondary antibodies Alexa-fluorophore conjugated antibodies (Invitrogen) and HRP-coupled secondary antibodies (MoBiTec) were used. For staining of DNA Hoechst 33258 (Invitrogen) was used.

Immunohistochemistry of cryosections

Embryonic brains were fixed by direct injection of 4% paraformaldehyde in 120 mM phosphate buffer, pH 7.4 (PBS) into the lateral ventricle of the brain followed by a fixation overnight at 4°C in 4% paraformaldehyde in PBS, equilibrated in 30% sucrose in PBS and embedded in Tissue-Tek. Cryosections (10 µm) were prepared, permeabilized with 0.3% Triton X-100 in PBS, quenched with 10 mM NH_4Cl, blocked with 5% goatserum and 5% bovine serum albumin in PBS and subjected to immunohistochemistry stainings with primary antibodies diluted in blocking solution. Images were collected by confocal microscopy using LSM software (Zeiss); image analysis was performed with the LSM software, Adobe Photoshop and the IMAGE J software.

Immunohistochemistry of cells in culture

NIH3T3 cells were transfected with Fugene6 according to manufacturers instructions. NIH3T3 cells as well as cells transfected via ex vivo electroporation were processed for immunohistochemistry by fixation with 4% paraformaldehyde in 120 mM phosphate buffer, pH 7.4 (PBS), permeabilized with 0.05% Triton X-100 in PBS, blocked with 10% fetal calf serum in PBS and subjected to immunohistochemistry stainings with primary antibodies diluted in blocking solution. Images were collected and processed in the same way as images from cryosections. Permanent cultures of neural stem cells were produced as previously described (Conti et al., 2005). Their neurogenic divison was induced as described (Qian et al., 1998) by cultivation in DMEM/F12 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 10ng/mL bFGF-2 and 1% N2 supplement (all from Invitrogen).

Immunoprecipitation and Western blot

HEK293T cells were grown in DMEM supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. They were transfected with Fugene6 (Roche) according to the instructions of the manufacturer. HEK 293T cells were lysed 48 h after transfection with lysis buffer 1 (2% Triton X-100 and Complete protease inhibitor cocktail (Roche) in PBS) for 30 min at 4°C. For immunoprecipitation of ubiquitinated proteins was performed as previously described (Schwamborn et al., 2007). Briefly, clasto-Lactacystin-β-lactone was directly added to the medium and after culture for 24h, cells were lysed in lysis buffer 2 (1% SDS in PBS) for 30 min at room temperature. Subsequently, the lysate was diluted 1:10 with PBS. Ubiquitinated proteins were precipitated for 4 h at 4°C with an anti-myc antibody and overnight with Protein-G agarose. Afterwards bound proteins were eluted by boiling in 1% SDS, and the immunoprecipitation was repeated with the same antibody. For brain lysates E14.5 brains were lysed with lysis buffer 1 for 30 min at 4°C and were then processed for immunoprecipitations as described above. When these lysates were used for RNA-end-labeling RNase-Inhibitor (Roche) was added.

Supplemental References

Conti, L., Pollard, S.M., Gorba, T., Reitano, E., Toselli, M., Biella, G., Sun, Y., Sanzone, S., Ying, Q.L., Cattaneo, E., and Smith, A. (2005). Niche-independent symmetrical self-renewal of a mammalian tissue stem cell. PLoS Biol. 3, e283. 10.1371/journal.pbio.0030283

Supplemental Figure Legends

Fig. S1 Trim32 inhibits proliferation in fibroblasts

(A) Domain organization of the *Drosophila* Brat protein and its mammalian ortholog TRIM32. Abbreviations: RING – really interesting new gene domain, B – B-Box; cc – coiled coil and NHL – Ncl1, HT2A and Lin41 homology domain.
(B, D and F) Immunostainings of NIH3T3 cells transfected as indicated (left grey boxes) and labeled with the indicated markers (upper grey boxes). Phospho-Histone H3 (P-H3) (B, C) is used to indicate mitotic activity, Ki67 (D, E) is used to show cell cycle engagement and Fibrillarin (F, G) visualizes the nucleolus. Arrows point to nuclei of transfected cells.
(C, E, and H) Diagrams show the fraction of transfected cells that are positive for Phospho-Histone H3(C), Ki67 (E) or Cleaved Caspase-3 (G) (mean +/- s.e.m.; *P<0.001 compared to EGFP).
(G) Diagram shows the relative size of Fibrillarin positive nucleoli in transfected cells. Avarage nucleolus size in EGFP transfected cells was set to one (mean +/- s.e.m.; *P<0.001 compared to EGFP).

Fig. S2 Expression of Trim32 in the developing brain

(A) Immunostainings of cryosections from the E12.5, E14.5 and E18.5 mouse cortex labeled with the indicated markers (left grey boxes).
(B) Immunostainings of cryosections from the E14.5 mouse cortex labeled with the indicated markers (upper grey boxes). The anti-TRIM32 antibody was blocked by preincubation with the immunogenic peptide.
(C) The diagram shows the fraction of neural progenitors at the indicated stages that show a significant enrichment of TRIM32 at the basal pole.
(D) Immunostainings of a single neural progenitor in a cryosection labeled with the indicated markers (upper grey boxes). The immunofluorescence intensity of TRIM32 and HSP70 at the apical pole and at the basal pole was determined. From these values the basal enrichment for each protein was calculated (basal fluorescence intensity divided by apical fluorescence intensity). The basal enrichment of Hsp70 was used to calculate the corrected relative Trim32 enrichment at the basal pole (Rel. Trim32 enrichment = Basal Trim32 entrichment / Basal Hsp70 enrichment).
(E, F, G and I) Immunostainings of cryosections from the E14.5 mouse cortex labeled with the indicated markers (upper grey boxes). Lower panels in H) show a high magnification of the ventricular zone (most apical).
(H) The diagram shows the distribution of the TRIM32-immunofluorescence intensity in the apical half and the basal half of cortical neural progenitor cells in anaphase.
(J) Immunostainings of cryosections from the E16.5 mouse cortex labeled with the indicated markers (upper grey boxes). Panels at the right show a high magnification of a mitotic basal progenitor.

Fig. S3 Trim32 regulates cell fate *in vitro*

(A and B) Neural progenitors were transfected at E14.5 via *in utero* electroporation with the indicated constructs (left grey boxes). The electroporation was followed by dissociation of the

tissue and cultivation for four days. Immunostainings of the dissociated cells labeled as indicated (upper grey boxes) are shown.
(C) The diagram shows the fraction of transfected cells that are positive for the neuronal maker MAP2 (mean +/- s.e.m.; *P<0.001 compared to EGFP).

Fig. S4 Trim32-RNAi constructs are functional

(A) HEK293T cells were transfected as indicated. The cell lysates were subjected to Western Blotting with the indicated antibodies.
(B) Neural progenitors were transfected at E14.5 via *in utero* electroporation with the indicated construct (left grey boxes). The electroporation was followed by dissociation of the tissue and cultivation for four days. Immunostainings of the dissociated cells labeled as indicated (upper grey boxes) are shown. The dashed green line in the middle panel highlights the transfected cell.
(C) Neural progenitors were transfected at E14.5 via *in utero* electroporation with the indicated constructs (upper grey boxes). The electroporation was followed by dissociation of the tissue and cultivation for ten days.

Fig. S5 Trim32 regulates cell fate *in vivo*

(A and B) Coronal cryosections of the cortex from embryonic brains at E15.5 (A) or E18.5 (B) that have been transfected as indicated (left grey boxes) at E14.5. The indicated constructs were transfected into the neural progenitors by *in utero* electroporation. The cells were labeled with the indicated markers (upper grey boxes).

Fig. S6 Proliferation regulation by Trim32 C24A

(A) HEK293T cells were transfected as indicated, incubated overnight with clasto-Lactacystin-β-lactone (β-Lactone) and Myc was immunoprecipitated twice (2xIP) with an anti-Myc antibody. Ubiquitin conjugated Myc (Myc-(Ubi)n) was detected with an anti-HA antibody. Myc and Trim32 in the lysate were detected with specific antibodies as indicated.
(B and C) Immunostainings of NIH3T3 cells transfected as indicated (left grey boxes) and labeled with the indicated markers (upper grey boxes). Arrows point to nuclei of transfected cells.
(D and E) The diagrams show the fraction of transfected cells that are positive for the mitotic marker Phosphorylated Histone-3 (P-H3) (D) and the proliferation antigen Ki67 (E) (mean +/- s.e.m.; *P<0.001 compared to EGFP).

Fig. S7 TRIM32 activates associated micro RNAs

Diagram showing the normalized activity of luciferase sensors for the TRIM32 associated micro RNAs (see Fig. 7) when being coexpressed with TRIM32. Luciferase sensor activity in control transfections with only EGFP was set to 100%.

Additional Supplemental Data

A complete report for the relative cloning frequencies for all microRNAs that were identified by massive parallel sequencing is provided (Deep Sequencing report.tgz). The individual immunoprecipitations are labeled as follows: JJ1: total RNA; JJ2: Control-IP; JJ3: Ago1-IP; JJ4: TRIM32-IP.

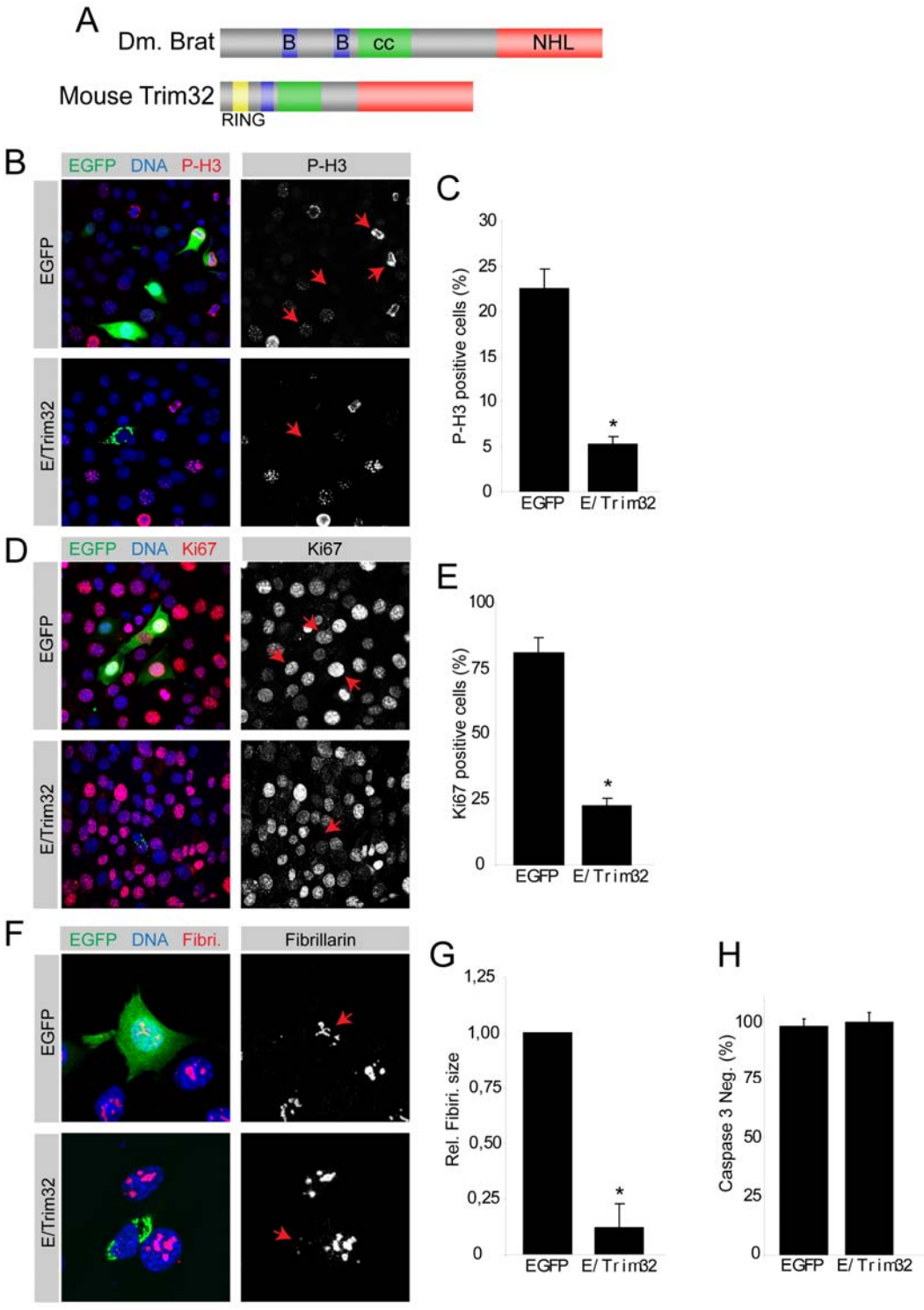
A
Dm. Brat
B
B
cc
NHL
Mouse Trim32
RING
B
EGFP DNA P-H3
P-H3
EGFP
E/Trim32
C
P-H3 positive cells (%)
30
25
20
15
10
5
0
*
EGFP
E/ Trim32
D
EGFP DNA Ki67
Ki67
EGFP
E/Trim32
E
Ki67 positive cells (%)
100
75
50
25
0
*
EGFP
E/ Trim32
F
EGFP DNA Fibri.
Fibrillarin
EGFP
E/Trim32
G
Rel. Fibiri. size
1,25
1,00
0,75
0,50
0,25
0
*
EGFP
E/ Trim32
H
Caspase 3 Neg. (%)
100
75
50
25
0
EGFP
E/ Trim32

Fig. S1

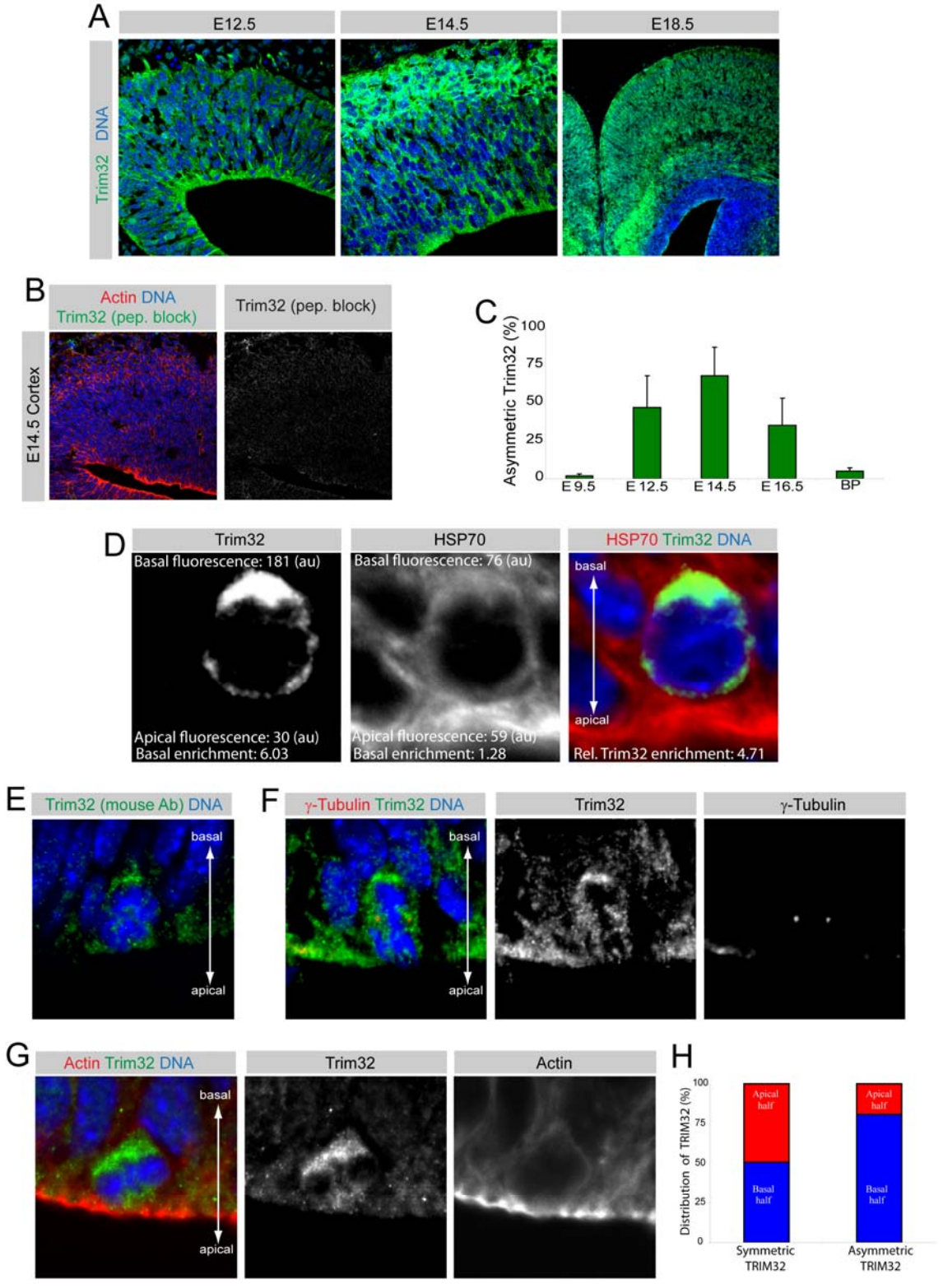
A
E12.5
E14.5
E18.5
Trim32 DNA
B
Actin DNA
Trim32 (pep. block)
Trim32 (pep. block)
E14.5 Cortex
C
Asymmetric Trim32 (%)
100
75
50
25
0
E 9.5
E 12.5
E 14.5
E 16.5
BP
D
Trim32
HSP70
HSP70 Trim32 DNA
Basal fluorescence: 181 (au)
Basal fluorescence: 76 (au)
basal
apical
Apical fluorescence: 30 (au)
Basal enrichment: 6.03
Apical fluorescence: 59 (au)
Basal enrichment: 1.28
Rel. Trim32 enrichment: 4.71
E
Trim32 (mouse Ab) DNA
basal
apical
F
γ-Tubulin Trim32 DNA
Trim32
γ-Tubulin
G
Actin Trim32 DNA
Trim32
Actin
H
Distribution of TRIM32 (%)
Apical half
Basal half
Symmetric TRIM32
Asymmetric TRIM32

Fig. S2

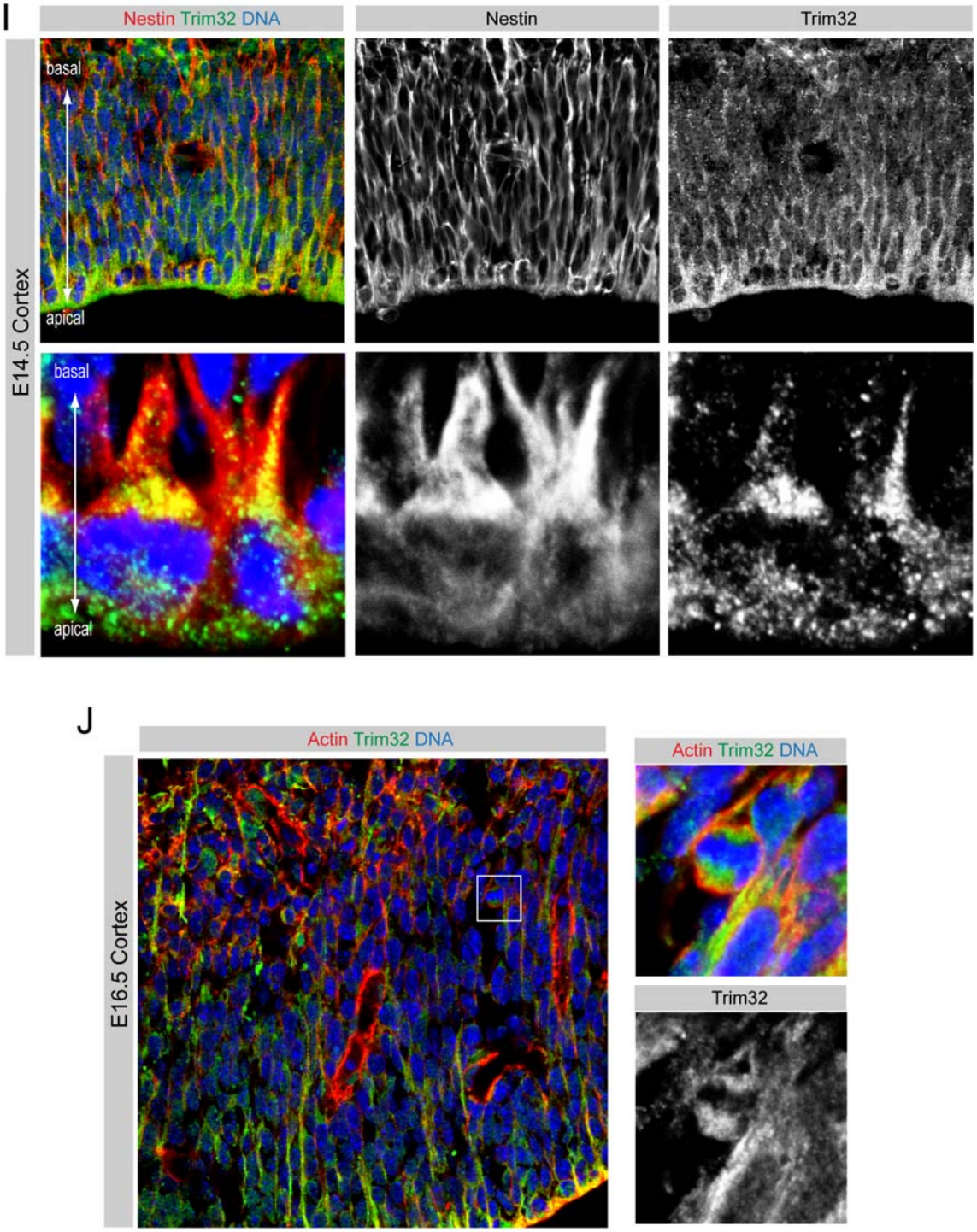
I
Nestin Trim32 DNA
Nestin
Trim32
E14.5 Cortex
basal
apical
basal
apical
J
Actin Trim32 DNA
E16.5 Cortex
Actin Trim32 DNA
Trim32

Fig. S2

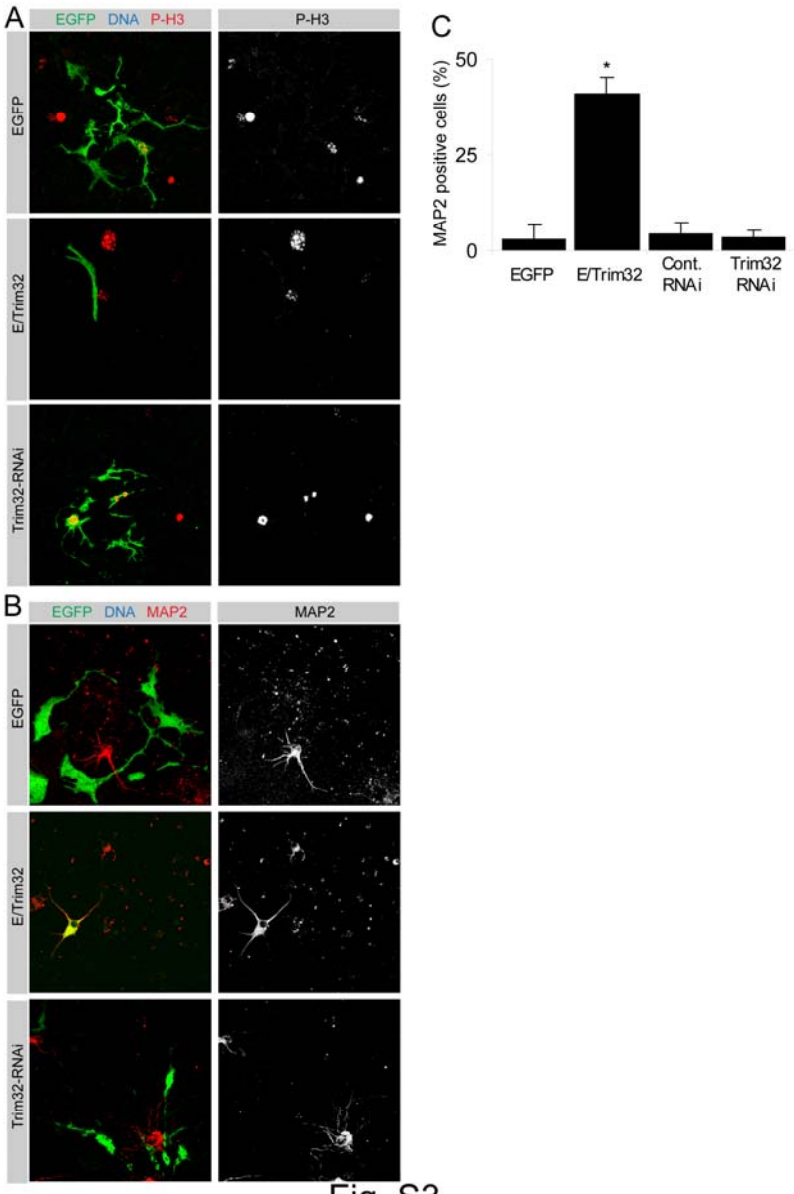
A
EGFP DNA P-H3
P-H3
EGFP
E/Trim32
Trim32-RNAi
B
EGFP DNA MAP2
MAP2
EGFP
E/Trim32
Trim32-RNAi
C
MAP2 positive cells (%)
50
25
0
*
EGFP
E/Trim32
Cont. RNAi
Trim32 RNAi

Fig. S3

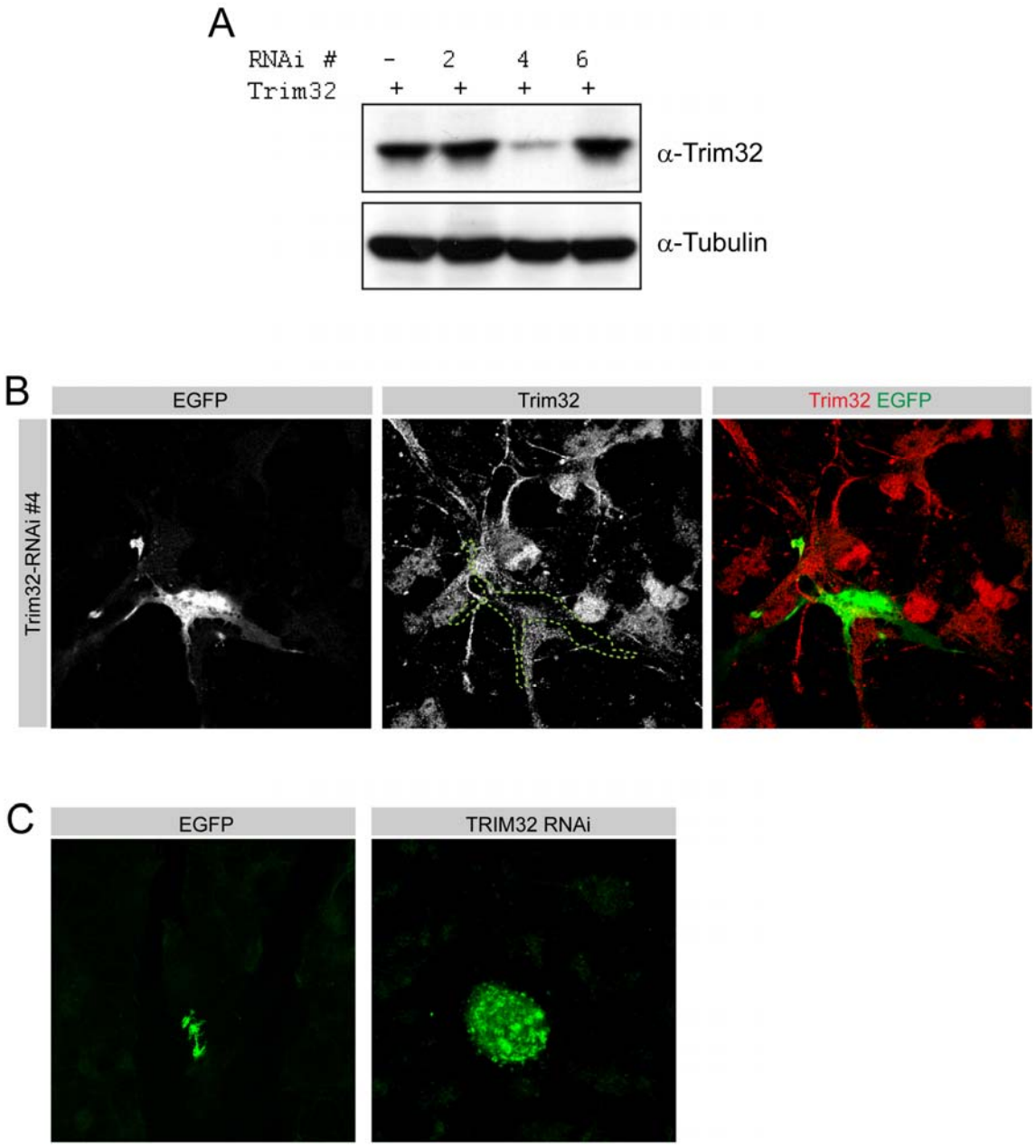
A
RNAi # - 2 4 6
Trim32 + + + +
α-Trim32
α-Tubulin
B
EGFP
Trim32
Trim32 EGFP
Trim32-RNAi #4
C
EGFP
TRIM32 RNAi

Fig. S4

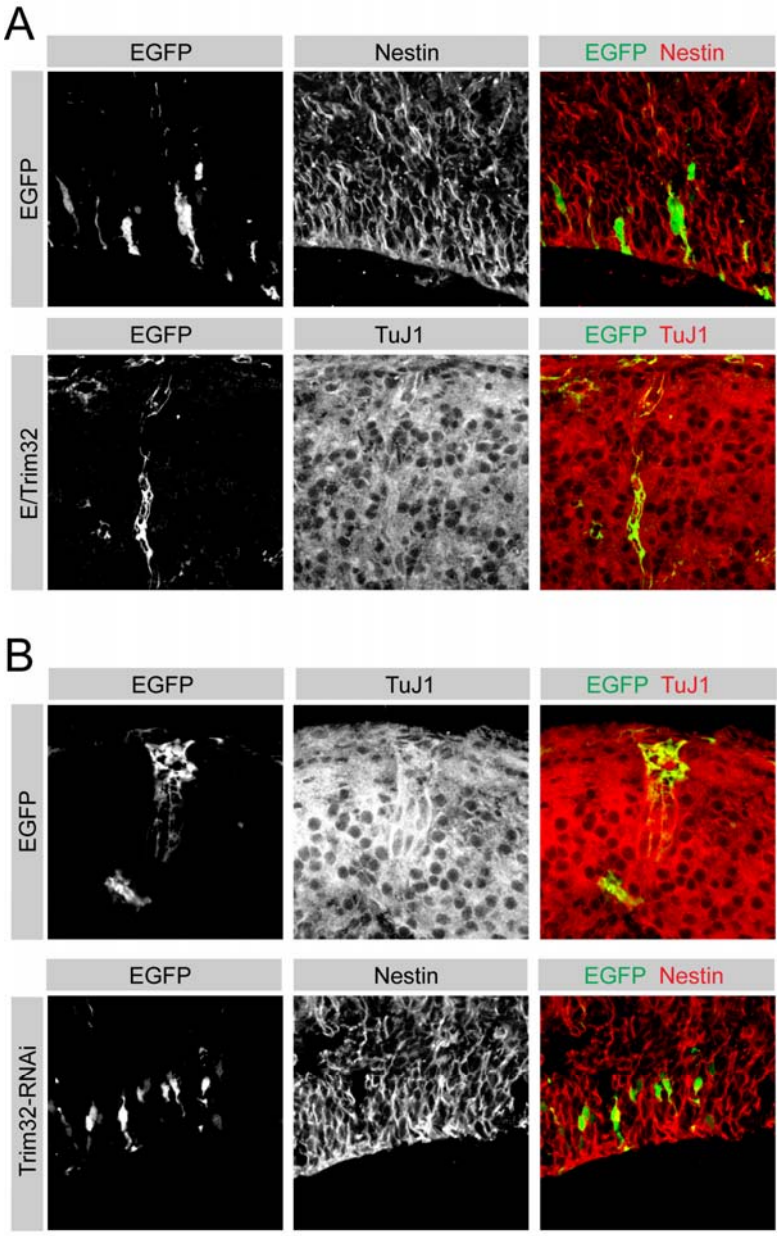
A
EGFP
Nestin
EGFP Nestin
EGFP
EGFP
TuJ1
EGFP TuJ1
E/Trim32
B
EGFP
TuJ1
EGFP TuJ1
EGFP
EGFP
Nestin
EGFP Nestin
Trim32-RNAi

Fig. S5

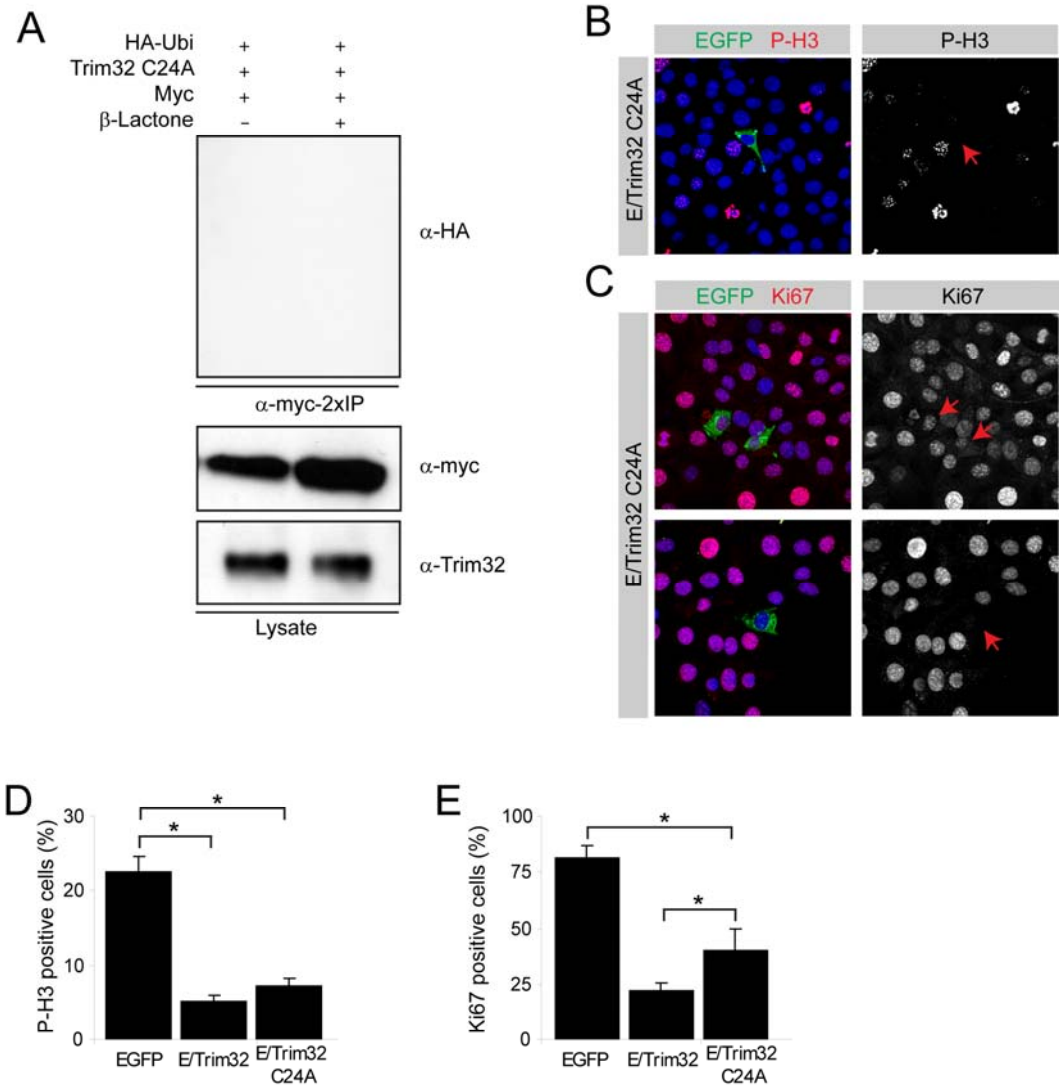
A
HA-Ubi + +
Trim32 C24A + +
Myc + +
β-Lactone - +
α-HA
α-myc-2xIP
α-myc
α-Trim32
Lysate
B
EGFP P-H3
P-H3
E/Trim32 C24A
C
EGFP Ki67
Ki67
E/Trim32 C24A
D
P-H3 positive cells (%)
30
20
10
0
*
*
EGFP
E/Trim32
E/Trim32 C24A
E
Ki67 positive cells (%)
100
75
50
25
0
*
*
EGFP
E/Trim32
E/Trim32 C24A

Fig. S6

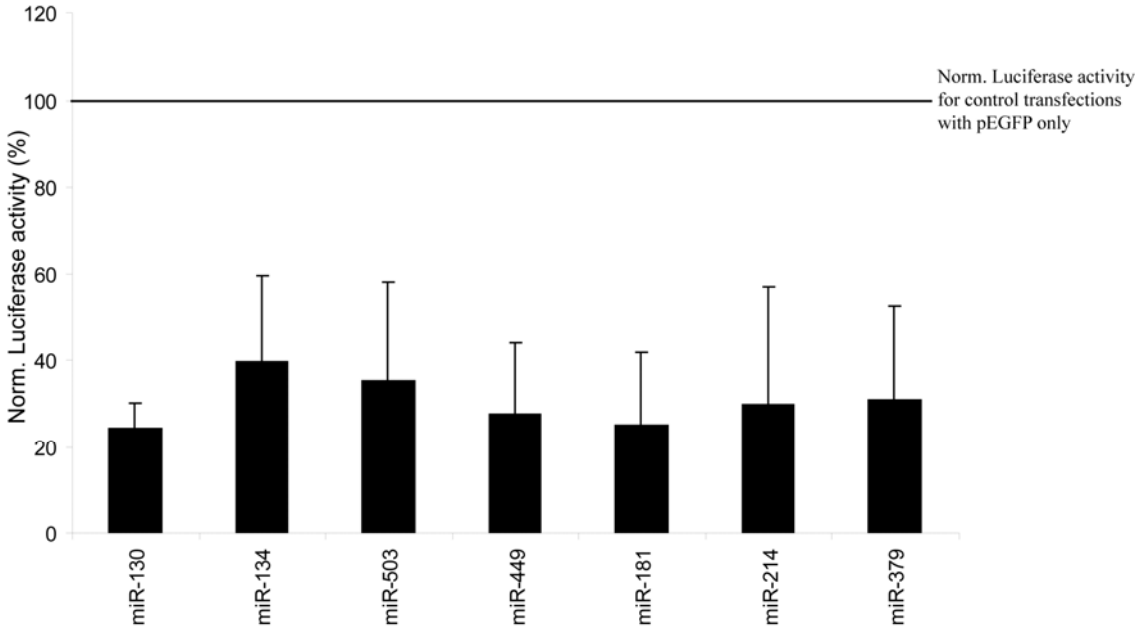
120
100
80
60
40
20
0
Norm. Luciferase activity (%)
Norm. Luciferase activity for control transfections with pEGFP only
miR-130
miR-134
miR-503
miR-449
miR-181
miR-214
miR-379

Fig. S7